RhoGDI2 Expression Is Associated with Tumor Growth and Malignant Progression of Gastric Cancer

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Abstract

Purpose: Rho GDP dissociation inhibitor 2 (RhoGDI2) has been identified as a regulator of Rho family GTPase. However, there is currently no direct evidence suggesting whether RhoGDI2 activates or inhibits Rho family GTPase in vivo (and which type), and the role of RhoGDI2 in tumor remains controversial. Here, we assessed the effects of RhoGDI2 expression on gastric tumor growth and metastasis progression.

Experimental Design: Proteomic analysis was done to investigate the tumor-specific protein expression in gastric cancer and RhoGDI2 was selected for further study. Immunohistochemistry was used to detect RhoGDI2 expression in clinical samples of primary gastric tumor tissues which have different pathologic stages. Gain-of-function and loss-of-function approaches were done to examine the malignant phenotypes of the RhoGDI2-expressing or RhoGDI2-depleting cells.

Results: RhoGDI2 expression was correlated positively with tumor progression and metastasis potential in human gastric tumor tissues, as well as cell lines. The forced expression of RhoGDI2 caused a significant increase in gastric cancer cell invasion in vitro, and tumor growth, angiogenesis, and metastasis in vivo, whereas RhoGDI2 depletion evidenced opposite effects.

Conclusion: Our findings indicate that RhoGDI2 is involved in gastric tumor growth and metastasis, and that RhoGDI2 may be a useful marker for tumor progression of human gastric cancer.

Tumor invasion and metastasis are the critical steps in determining the aggressive phenotype of human cancers, and also constitute the principal causes of cancer deaths (1). Rho GTPases, including RhoA, Rac1, and cdc42, control a wide range of signaling pathways that regulate a variety of biological processes. Aberrant signaling through these proteins, which is commonly observed in human cancers, has been implicated in facilitating virtually all aspects of the malignant phenotype (2, 3).

The biological activities of Rho GTPases are controlled through a tightly regulated GDP/GTP cycle, which is stimulated by guanine nucleotide exchange factors and terminated by GTPase-activating proteins (4). An additional level of regulation is provided by Rho GDP dissociation inhibitors (RhoGDI; ref. 5). RhoGDI binds the majority of Rho GTPases in the cytoplasm, maintaining Rho in an inactive form in which it cannot interact with effector target proteins (6). On the other hand, RhoGDI also associated with active forms of Rho, Rac, and cdc42 (7, 8). This interaction resulted in an inhibition of the intrinsic and GTPase-activating protein–stimulated GTPase activities of the Rho GTPases, maintaining Rho in an active form. Three human RhoGDIs have been identified thus far: RhoGDI1 (also referred to as RhoGDI or RhoGDI-a), RhoGDI2 (Ly-GDI or D4GDI or RhoGDI-β), and RhoGDI3 (RhoGDI-γ; refs. 9–11). RhoGDI2 is preferentially expressed in hematopoietic cells, and RhoGDI3 is expressed in the brain, lungs, kidneys, testes, and pancreas, whereas RhoGDI1 is ubiquitously expressed in all mammalian organs (12, 13).

Several reports have assessed the expression levels of RhoGDIs in a variety of cancer cells, revealing opposite patterns depending on the tumor type. RhoGDI1 is up-regulated in ovarian and breast cancers (14, 15). By way of contrast, the down-regulation of RhoGDI1 has been also reported in cases of breast cancer (16). The mRNA level of RhoGDI2 is significantly higher in ovarian adenocarcinoma than in benign adenoma (17). Consistent with this, a recent report showed that RhoGDI2 is overexpressed in human breast cancer cell lines and increases cancer cell invasion and motility in vitro (18). By
Human RhoGDI2 cDNA was amplified by PCR using the following two primers with 10% fetal bovine serum and antibiotics. The amplified product was maintained in DMEM-F12 medium (Life Technologies) supplemented with 10% nonfat dry milk, and proved overnight at 4°C with the appropriate primary antibodies. The bound antibodies were visualized with a suitable secondary antibody conjugated with horseradish peroxidase using enhanced chemiluminescence reagents (Amersham Bioscience). We purchased antibodies specific for RhoGDI2 (Spring Bioscience), RhoGDI1 (Santa Cruz Biotechnology), and α-tubulin (Sigma).

**Western blot analysis.** Cells were lysed in a lysis buffer [20 mmol/L Tris (pH 7.4), 2 mmol/L EDTA, 1% Triton X-100, 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, and 10 μg/mL leupeptin]. The cell lysates were separated by 7.5% to 15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Amersham Bioscience). Subsequently, the membrane was incubated for 1 h at room temperature in a TBS solution [10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, and 0.05% Tween 20] supplemented with 5% nonfat dry milk, and probed overnight at 4°C with the appropriate primary antibodies. The bound antibodies were visualized with a suitable secondary antibody conjugated with horseradish peroxidase using enhanced chemiluminescence reagents (Amersham Bioscience). We purchased antibodies specific for RhoGDI2 (Spring Bioscience), RhoGDI1 (Santa Cruz Biotechnology), and α-tubulin (Sigma).

**RNA interference experiments.** Two different small interfering RNA (siRNA) oligo duplexes for targeting RhoGDI2 genes were purchased from Samchul. The sequence was as follows: siGDI2-A: 5′-GAUGAGAGCUCUAUAAUAGT-3′, siGDI2-B: 5′-GGAAGGCUUCAAAUAAUC-3′. Transient transfection of each siRNA oligo duplex was accomplished using LipofectAMINE Plus Reagent (Invitrogen) following the instructions of the manufacturer. After incubation for 48 h, the cells were harvested and the efficiency of each siRNA oligo duplex was confirmed by Western blotting using anti-RhoGDI2 antibody. The short hairpin RNA (shRNA)—expressing lentiviral vector for targeting the RhoGDI2 gene was constructed by inserting synthetic double-stranded oligonucleotides (5′-CGGAATTCCAGGATGAGAGGATTCGATCGGCTGAGCTGATTCATTATTCGACATCTTTTCTTTGATATCGATCTAGACA-3′) into the shLenti2.4G lentiviral vector. As a control vector, a scrambled siRNA (5′-AATCCGATACGGTGATCCGGT-3′), which has no homology with the mammalian mRNA sequences, was inserted into the shLenti2.4G lentiviral vector as described above. shRNA expression vector was transected into lentiviral packaging cell lines 293T cells. The culture supernatant containing virus particles was harvested 48 h after transfection, clarified with a 0.45 μmol/L membrane filter (Nalgene). For stable transduction of lentivirus containing shRNA expression vector, cells at 60% to 70% confluency were used. After 48 h, 1 μg/mL puromycin (Clontech) was added to the cultures for selection. After 14 days, puromycin-resistant cell pools were established.

**Invasion assay.** Cell invasion was done using QCM 24-well Cell Invasion Assay kit (Chemicon) following the instructions of the manufacturer. Cells (2.5 × 10⁴) in 250 μL of medium were placed in the insert and allowed to invade for 20 h. The lower chamber was filled with 500 μL of the appropriate medium containing 20% fetal bovine serum. After incubation, cells/medium remaining on top of the insert were removed by pipetting. The invasion chamber insert was transferred into a clean well containing 225 μL of prewarmed Cell Detachment Solution, and incubated for 30 min at 37°C. The insert was removed from the well. Lysis buffer/dye solution (75 μL; CyQuant GR Dye 1:75 with 4X lysis buffer) was added into each well and incubated for 15 min at room temperature. Two hundred microliters of the mixture was transferred into a 96-well plate and assessed with a fluorescence plate reader using a 480/520 nm filter set.

**Proliferation assay.** The cells were placed in a six-well plate at a concentration of 3 × 10⁴ cells per well. After incubation for 1 to 4 days, the viable cells were counted with a hemocytometer after trypan blue staining.

**Tumor growth and metastasis in mice.** All animal experiments were approved by the Institutional Animal Care and Use Committees of Ewha Womans University and followed National Research Council Guidelines. Eight-week-old male BALB/cScid-nude mice were purchased from SLC, Inc. (Japan). All mice were maintained in the Ewha way of contrast, RhoGDI2 expression is correlated inversely with invasive capacity in bladder cancer cell lines (19). Furthermore, the reduced expression of the RhoGDI2 protein has been associated with the poor prognosis of patients with advanced bladder cancer (20). Thus, the role of RhoGDI2 in tumor progression remains controversial.

In this study, we aim to uncover the putative role of RhoGDI2 in the carcinogenesis of gastric cancer. By comparing the expression profile of RhoGDI2 protein in cancer and noncancerous tissues, and characterizing the role of this gene in regulating tumor growth and metastasis both in vitro and in vivo, we report that RhoGDI2 functions as an activator of tumor growth and malignant progression in gastric cancer.

**Materials and Methods**

**Cell culture.** Human gastric cancer cell lines MKN-28, MKN-45, SNU-1, SNU-16, SNU-484, SNU-638 and SNU-719 were obtained from the Korean Cell Line Bank (Seoul, Korea). Human breast cancer cell lines MCF-7 and MDA-MB-231 were purchased from American Type Culture Collection. The human gastric cancer cell lines were maintained in RPMI 1640 (Life Technologies) and breast cancer cell lines were maintained in DMEM-F12 medium (Life Technologies) supplemented with 10% fetal bovine serum and antibiotics.

**Construction of the RhoGDI2 expression plasmid and transfection.** Human RhoGDI2 cDNA was amplified by PCR using the following two sets of primers: 5′-GATCGTAAAGAGGCCCAAC-3′ and 5′-GATCGTAAAGAGGCCCAAC-3′. PCR products were cloned into the Flag-tagging expression vector, pCMVTag-2B/Neomycin (Invitrogen). SNU-484 and SNU-719 cells were transfected with RhoGDI2-expressing plasmid by using LipofectAMINE reagent (Life Technologies) following the instructions of the manufacturer. After 48 h of incubation, cells were treated with G418 for selection.

**Total RNA extraction and reverse transcription-PCR.** Total RNA was extracted from the cultured cells using RNAeasy Mini Kit (Qiagen) following the instructions of the manufacturer. Reverse transcription-PCR was done using a Maxime RT-PCR PreMix kit (Intron). The PCR primers used for the amplification of RhoGDI2 mRNA were as follows: 5′-ATGACTGAAAAGGCCCAAC-3′ and 5′-GATCTGTCCTCCATCTCTCT-3′. The amplification of β-actin was used as an internal control.

**Translational Relevance**

Gastric cancer is one of the most common malignancies worldwide and ranks second in terms of global cancer-related mortality. The acquisition of an invasive phenotype is a prerequisite for the metastatic spread of tumor cells, which constitutes a major cause of poor prognosis for patients with cancer. In this study, we reveal an essential role of Rho GDP dissociation inhibitor 2 (RhoGDI2), which is usually known as a negative regulator of Rho family GTPase and potential suppressor of tumor metastasis, in promoting gastric cancer invasiveness and metastasis in vitro and in vivo. Our report also suggests that RhoGDI2 merits investigation as a prognostic marker for gastric cancer. Thus, we suggest that the interruption of the RhoGDI2-induced gastric cancer invasion and metastasis by an interferential inhibitor may prove to be a powerful therapeutic approach for interventions in cases of human gastric cancer.
Laboratory Animal Genomics Center under specific pathogen–free conditions. For tumor growth experiments, SNU-484(Mock), SNU-484(GDI2-4), and SNU-484(GDI2-6) cells (5 × 105/mouse) were injected s.c. into BALB/cSlc-nude mice (n = 8 per group). Tumors were measured with calipers to estimate volume (0.5 × width2 × length) from day 18 to day 35 after injection. After determination of tumor volume, excised tumors and metastatic lungs were fixed with parafomaldehyde (4%, 18 h, 4°C) and postfixed (70% ethanol, 16 h) before dehydration and paraffin embedding. Paraffin sections were stained with H&E. For lung metastasis assay, SNU-484(Mock), SNU-484(GDI2-4), and SNU-484(GDI2-6) cells (1 × 106/mouse in 100 μL of PBS) were injected into the tail vein of recipient mice (n = 8 per group). Six weeks after cell injection, lung tissues were excised. Lung metastasis was quantified by counting metastatic lesions in each section (10 sections per lung). For blood vessel evaluation, primary tumors were immunostained with anti-CD31 antibody (1:50; AngioBio).

**Patients, specimens, and immunohistochemistry procedure.** From the institution's surgical database, we randomly selected 26 gastric cancer patients who were operated in the Gyeongsang National University Hospital. The formalin-fixed, paraffin-embedded blocks of the enrolled patients were sectioned at 4 μm and mounted on charged slides. After the deparaffinization process with 60°C heat followed by xylene and graded alcohol, tissues were immunohistochemically stained with diluted primary antibody against RhoGDI2 (1:200; Spring Bioscience) using a LSAB kit (DAKO). Antigen retrieval was facilitated by microwaving the tissue for 30 min, and the rest of the staining procedure followed the standard avidin-biotinylated peroxidase complex method. RhoGDI2 expression in gastric cancer cells were compared with normal gastric tissue and was graded as positive when >30% of the cancer cells were strongly stained. Immunohistochemical staining was scored by a board-certified pathologist (D.C. Kim). This scoring was carried out in a blinded fashion, without knowledge of the patient follow-up information.

**Statistical analysis.** Data are presented as mean with 95% confidence intervals of at least three independent experiments. Different values among groups were compared using Student’s t test. All statistical tests were two-sided, and P < 0.05 was considered to be statistically significant.

**Results**

**RhoGDI2 expression in gastric tumor tissues.** We previously conducted a proteomic analysis with 152 human gastric tumor tissues to identify differentially expressed proteins in gastric tumor tissues as compared with normal tissues (21). Through our proteomic study, we observed a significant up-regulation of RhoGDI2 proteins in gastric tumor tissues (2.34 times, P < 0.001) compared with their corresponding normal gastric tissues (Fig. 1A and B). To ascertain whether the expression of RhoGDI2 can be correlated with certain pathologic phenotypes in clinical gastric tumor samples, we assessed the expression of RhoGDI2 in 26 cases of surgically removed human gastric tumor tissues using a monoclonal antibody specific to RhoGDI2. Strong staining for the RhoGDI2 was frequently observed in the nucleus of tumor cells of advanced stage gastric carcinomas, which invaded into the subserosa (Fig. 1C, right; Table 1), although it was also occasionally seen in the cytoplasm. By way of contrast, negative or very weak RhoGDI2 staining was observed in early stage gastric cancer tissues (Fig. 1C, middle; Table 1) and normal gastric tissues (Fig. 1C, left). The specificity of the antibody’s recognition of RhoGDI2 was verified using normal mouse IgG controls (data not shown). One important finding was that frequent RhoGDI2 expression is correlated with lymph node metastasis. Patients whose tumors expressed RhoGDI2 were more likely than those that had tumors not expressing RhoGDI2 to have advanced lymph node metastasis (Table 1).

**RhoGDI2 expression in gastric cancer cell lines.** In order to assess the functions of RhoGDI2, we compared the expression of RhoGDI2 proteins in different human gastric cancer cell lines. Interestingly, RhoGDI2 was expressed only in the cancer cell lines derived from the secondary tumor sites (ascites), SNU-16 and SNU-638, but not in the primary cancer cell lines, SNU-1, SNU-484, and SNU-719 (Fig. 2A; refs. 22, 23). SNU-638 cells have been known to be more invasive than SNU-484 cells (24). RhoGDI2 was also expressed in the highly invasive gastric cancer cell line, MKN-28, but not in MKN-45 cells, which evidence poor invasive ability (Fig. 2A; ref. 25). As has been reported, the RhoGDI2 protein was also expressed only in MDA-MB-231, a highly invasive breast cancer cell line, but not in the poorly invasive MCF-7 cells (Fig. 2A; ref. 18). However, we were unable to detect any critical differences in RhoGDI2 expression in all of these cell lines (Fig. 2A). In addition, the level of RhoGDI2 mRNA expression in each cell coincided precisely with that of the proteins (Fig. 2B). All of these findings led us to hypothesize that RhoGDI2 expression could influence the malignant progression of human gastric cancer cells.

**Overexpression of RhoGDI2 enhances gastric cancer cell invasion in vitro.** In an effort to determine whether RhoGDI2 expression is associated with the invasiveness of gastric cancer cell lines, we established RhoGDI2-overexpressing cells from SNU-484 and SNU-719 cells (Fig. 3A), as these parental cell lines do not authentically express RhoGDI2 (Fig. 2A). The forced expression of RhoGDI2 caused a significant increase in the invasiveness of each cell line as compared with the vector-transfected control cells (Fig. 3B). In an effort to exclude the possibility that the effect of RhoGDI2 on invasiveness was attributable to different proliferation rates, we compared the growth rates of RhoGDI2-overexpressing cells with those of control cells. All of these cells evidenced similar growth rates (Fig. 3C), thereby indicating that an increase of tumor cell invasiveness via the expression of RhoGDI2 in respective cells is not associated with the proliferation rates.

**Depletion of RhoGDI2 expression suppresses gastric cancer cell invasion in vitro.** We also assessed the effects of RhoGDI2 depletion on tumor cell invasion in RhoGDI2-depleting SNU-638 and MKN-28 cells via the use of Lenti.RhoGDI2-shRNA. Before establishing the RhoGDI2-depleting cells, we confirmed the specificity of two different RhoGDI2 siRNAs to down-regulate RhoGDI2 gene expression (Supplementary Fig. S1A). We also evaluated the specificity of Lenti.RhoGDI2-shRNA to down-regulate RhoGDI2 gene expression. Lenti.RhoGDI2-shRNA, but not Lenti.Con-shRNA expressing a scrambled oligonucleotide, was shown to repress RhoGDI2 expression in each cell line (Fig. 4A). However, Lenti.RhoGDI2-shRNA did not influence RhoGDI1 expression (Fig. 4A). RhoGDI2 depletion caused a significant reduction in the invasiveness of each cell line (Fig. 4B; Supplementary Fig. S1B). The proliferation rates of RhoGDI2-depleting cells were not significantly different with those of control cells (Fig. 4C). Depletion of RhoGDI2 also markedly suppressed the invasive ability of the MDA-MB-231 breast cancer cell
line (Supplementary Fig. S2), as previously reported (18). All of these results suggest that RhoGDI2 positively regulates the invasiveness of gastric cancer cells as well as breast cancer cells.

**RhoGDI2 promotes tumor growth, angiogenesis, and metastasis in vivo.** To assess the effects of RhoGDI2 on tumor growth in vivo, we implanted the RhoGDI2-expressing SNU-484 [SNU-484(GDI2-4) and SNU-484(GDI2-6)] and control [SNU-484(Mock)] cells subcutaneously into nude mice, and monitored the growth of the resultant primary tumors. Palpable tumors were first detected in all mice by day 18 after injection. At day 35, tumors in mice injected with SNU-484(GDI2-4) and SNU484(GDI2-6) cells were larger than tumors in mice injected with SNU-484(Mock) cells (Fig. 5A), whereas the in vitro growth rates of these cell lines did not differ significantly (Fig. 3C). Staining with anti-CD31 revealed that microvessel density was higher in the xenograft tumors derived from RhoGDI2-expressing SNU-484 cells than those derived from control cells (Fig. 5B). These results compelled us to assess lung metastasis, and we found that lung metastasis was observed in only one of eight mice (13%) injected with control cells, whereas it was detected in five of eight (63%) and six of eight mice (75%) injected with RhoGDI2-expressing SNU-484(GDI2-4) and SNU-484(GDI2-6) cells, respectively (Fig. 5C). We also found that numerous metastatic lung nodules were observed in mice injected with RhoGDI2-expressing cells, whereas a few nodules were detected in mice injected with control cells (Fig. 5D). To further test the effect of RhoGDI2 expression on metastasis, we

**Fig. 1.** Differential expression of RhoGDI2 in primary gastric specimens. **A** and **B,** noncancerous and cancerous tissues of 152 patients with gastric cancer were analyzed by two-dimensional electrophoresis. Image of a silver-stained two-dimensional electrophoresis gel after separation of proteins from a cancerous tissue (**A**). Zoomed regions of gel images from cancerous tissues and respective noncancerous tissues of four patients with gastric cancer (**B**). RhoGDI2 expression is increased in all cancerous tissues (dotted rectangles) compared with noncancerous tissues (closed rectangles). The images of silver-stained gels were analyzed with the software PDQuest. **C,** immunohistochemical staining of human gastric tumor tissues with RhoGDI2 antibody. The paraffin-embedded blocks of gastric tissue (normal tissue), early stage gastric cancer (ESGC, tumor stages I and II), and advanced stage gastric cancer (ASGC, tumor stages III and IV) were stained with a mouse monoclonal antibody against RhoGDI2 (brown) and counterstained with hematoxylin (blue). One representative image from 6 slides (normal tissue), 12 slides (ESGC), and 14 slides (ASGC), respectively.
injected RhoGDI2-expressing SNU484 and control cells into nude mice through the tail vein. We detected no metastasis in mice injected with control cells, whereas it was detected in four of eight mice injected with SNU-484(GDI2-4) and SNU-484(GDI2-6) cells, respectively (Supplementary Fig. S3). These results, collectively, indicate that RhoGDI2 expression profoundly increases tumor growth, angiogenesis, and metastasis in vivo.

**Discussion**

RhoGDI2 has been shown to be expressed only in hematopoietic tissues (10, 26). However, accumulating evidence suggests that RhoGDI2 is also expressed in the cells of nonhematopoietic neoplasms, including bladder, ovarian, and breast cancer (17, 18, 27). RhoGDI2 has been shown to be expressed in the T24 human bladder cancer cell line, but not in the more aggressive T24T lineage (27). In addition, the reduced expression of RhoGDI2 proteins was determined to be associated with poor prognosis in patients with advanced bladder cancer (19, 20). Consequently, it has been suggested that RhoGDI2 is a metastasis suppressor gene in bladder cancer. However, in this study, we have uncovered evidence suggesting that RhoGDI2 can function as a positive regulator of tumor progression in gastric cancers. First, we showed that the expression level of RhoGDI2 protein is much higher in gastric tumor tissues than in normal gastric tissues through proteomic analysis. Immunohistochemical results also showed that the expression level of RhoGDI2 is increased at higher gastric tumor stage and there is a significant relationship between RhoGDI2 expression and lymph node metastasis. The

**Table 1. Relationship between histology and RhoGDI2 expression**

<table>
<thead>
<tr>
<th>Tumor stage</th>
<th>No. of cases</th>
<th>RhoGDI2 positive cases (%)</th>
<th>P*</th>
</tr>
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<tbody>
<tr>
<td>I + II</td>
<td>12</td>
<td>0 (0)</td>
<td>0.016</td>
</tr>
<tr>
<td>III + IV</td>
<td>14</td>
<td>8 (57)</td>
<td></td>
</tr>
<tr>
<td>Nodal metastasis</td>
<td>26</td>
<td>8 (31)</td>
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</tr>
</tbody>
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*N*= 0.05 were considered to be statistically significant (stages III and IV vs. stages I and II; N1, N2 and N3 vs. N0).

**Fig. 2.** RhoGDI2 is overexpressed in metastatic and highly invasive gastric cancer cell lines. A, analysis of RhoGDI2 protein expression in human cancer cell lines. Cell lysates, as indicated, were subjected to immunoblotting analysis using antibodies specific for RhoGDI2 or RhoGDI1. Equal loading was confirmed by reprobing the membrane with antibodies to α-tubulin. One representative image of three independent experiments. B, analysis of RhoGDI2 mRNA expression in human cancer cell lines. The RhoGDI2 mRNA expression was determined by reverse transcription-PCR analysis. The amplification of β-actin was used as an internal control.

**Fig. 3.** Overexpression of RhoGDI2 promotes gastric cancer cell invasion. A, SNU-484 and SNU-719-derived cell lines stably transfected with empty (Mock) or RhoGDI2-overexpressing vector (GDI2-4 and GDI2-6 in SNU-484, GDI2-1 and GDI2-8 in SNU-719 cells) were analyzed by immunoblotting with antibody to RhoGDI2. B, effect of RhoGDI2 overexpression on in vitro invasion ability of SNU-484 and SNU-719 cells. The invasion activity of each clone was measured in vitro with the Boyden chamber after 20 h. Invasion of RhoGDI2-overexpressing cells was markedly increased compared with that of control cells (*, P < 0.05). C, effect of RhoGDI2 overexpression on the proliferation of SNU-484 and SNU-719 cells. Cell proliferation was assessed by counting the cells after trypan blue staining at each time point. We repeated the experiments in (B) and (C) three times, each in triplicate.
localization of RhoGDI2 in gastric tumor tissues was different with that in other tissues. RhoGDI2 has been known to be predominantly localized in the cytoplasm, and occasionally in the nucleus (20). However, in our study, RhoGDI2 was frequently observed in the nucleus of tumor cells of gastric cancer tissues (Fig. 1C). This discrepancy may suggest different roles for RhoGDI2 in tumor progression of gastric cancer, compared with other cancers. Second, RhoGDI2 is expressed only in metastatic and highly invasive gastric cancer cell lines. Forced expression of RhoGDI2 increases cancer cell invasion, whereas depletion of RhoGDI2 evidences opposite effects. Lastly, forced expression of RhoGDI2 significantly increases tumor growth, angiogenesis, and lung metastasis in vivo. Although RhoGDI2 did not affect the cancer cell proliferation in vitro, it enhanced the tumor volume in vivo. This discrepancy may be due to a secondary effect via increased tumor angiogenesis of RhoGDI2-expressing cells (Fig. 5B), as it has been known that tumor angiogenesis factor could promote tumor growth in vivo (28). Consistent with our findings, there is a report showing that RhoGDI2 promotes the invasiveness of breast cancer cells (18). All of these results indicate that RhoGDI2 may play an opposite role in the progression of different tumor types.

Although the reason for this discrepancy currently remains unclear, one plausible reason may be the dual roles of RhoGDIs in the regulation of Rho GTPase activities during cancer progression. RhoGDIs were originally identified as negative regulators of Rho GTPases. When exogenously introduced into cells, RhoGDI induces the disruption of Rho-dependent cellular activities, including cytoskeletal organization and motility (29). However, recent evidence points to a positive role for RhoGDIs in Rho GTPases' function. RhoGDIs could act as an escort protein directing Rho GTPases to the membrane, which is crucial for coupling the GTPases to their downstream effector proteins. For instance, Rac1 regulation of NADPH oxidase activity in neutrophils requires a complex with RhoGDI (30). Similarly, cdc42-mediated cellular transformation also requires not only the binding of activated cdc42 to RhoGDI but also the recruitment of cdc42 by RhoGDI to a specific cellular site in NIH-3T3 fibroblast cells (31). These results have led us to attempt to verify the activation status of RhoA, Rac1, and cdc42 in RhoGDI2-expressing and RhoGDI2-depleting cells, and found that RhoGDI2 specifically activates Rac1 in gastric cancer cells (our unpublished results). It is conceivable that RhoGDI2 may function as a positive regulator of Rho GTTPase signaling in gastric cancer cells. However, how RhoGDI2 causes the activation of Rac1 in gastric cancer cells requires further analyses. Another plausible scenario is that RhoGDI2 influences other signaling pathways. Groysman et al. showed that RhoGDI2 associates with Vav1 (the guanine nucleotide exchange factor of Rho GTTPases) and enhances, rather than counteracting, the effects of Rac1, the induction of NFAT during T-cell activation by T-cell receptor (32). This suggests that these two Rho GTTPase regulators seem to function cooperatively as signal transducers in the T-cell receptor signaling pathway. Recently, Schunke et al. also showed that RhoGDI2 cooperates with Vav1 for NFAT activation in breast cancer cells (33).

RhoGDI2 could modulate cancer cell invasion through its effect on the expression of β1-integrin, an integrin that plays a role in breast cancer progression. The expression of β1-integrin has been known to be regulated by RhoGDIs in breast cancer cells (18). Cyclooxygenase-2, a major regulator of invasion and metastasis in breast cancer cells (34—36), has also been identified as a target gene of RhoGDI2 (33). We attempted to verify whether the expression of β1-integrin or cyclooxygenase-2 is regulated by RhoGDI2 in gastric cancer cells, but failed to see any difference in the expression of these proteins in RhoGDI2-expressing or RhoGDI2-depleting gastric cancer cells compared with control cells (data not shown). These results may reflect the diversity of genetic backgrounds in different tumor cells. On the other hand, we identified many differentially expressed genes in RhoGDI2-expressing and RhoGDI2-depleting gastric cancer cells via microarray analysis (our unpublished results). Among these genes, we are currently focusing our attention on vascular endothelial growth factor-C as an alternative target for RhoGDI2 in gastric cancer cells because vascular endothelial growth factor-C has been shown to
promote tumor metastasis via an enhancement of tumor invasion, angiogenesis, and lymphangiogenesis (37–39), and RhoGDI2-expressing gastric cancer cells exhibited identical phenotypes in vivo in our study (Fig. 5).

In conclusion, our study shows that RhoGDI2 expression correlates with gastric cancer progression and metastasis. RhoGDI2 may prove to be an important molecule in understanding the biology of the carcinogenesis and metastasis of gastric cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
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References

Fig. 5. RhoGDI2 enhances tumor growth, angiogenesis, and lung metastasis in vivo. A, RhoGDI2 expression and tumor formation. Mice were injected s.c. with SNU-484 cells stably transfected with empty (Mock, n = 8) or RhoGDI2-expressing vector (GDI2-4 and GDI2-6, n = 8), and tumor size was measured over time. B, angiogenesis in xenograft tumors. Blood vessels in the primary tumor sections of each mouse described in (A) were prepared and stained with an anti-CD31 antibody (left). Brown, microvessels in the primary tumors. One representative staining image from 24 slides (SNU-484(Mock), SNU-484(GDI2-4), and SNU-484(GDI2-6), three per mouse), respectively. Number of CD31-positive vessels per field (right). Number of CD31-positive vessels in individual mice was counted under the microscope. Significantly increased number of metastatic lung nodules were observed in mice injected with RhoGDI2-expressing SNU-484 cells compared with those derived from control cells (**, P < 0.01).
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